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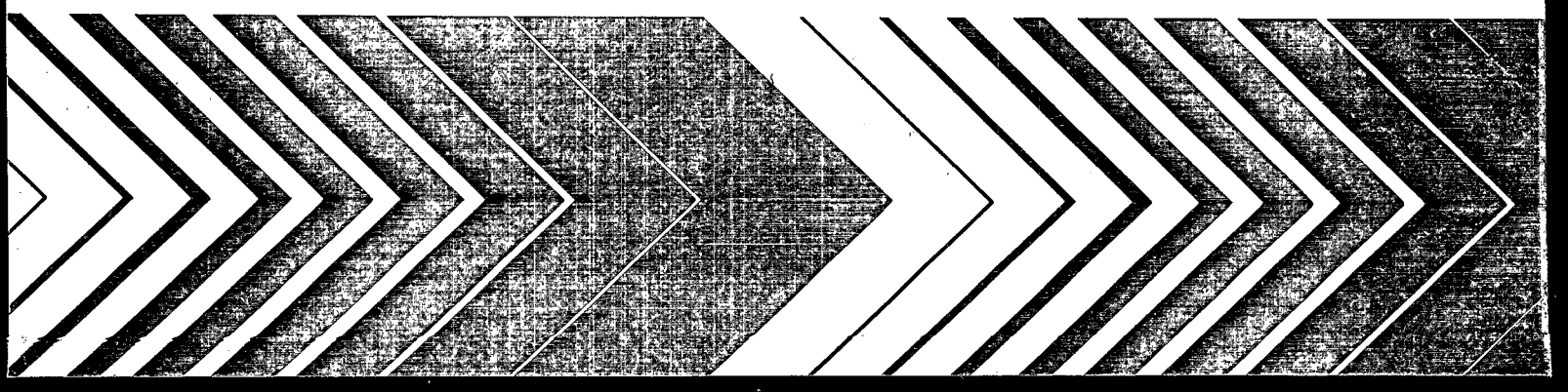
# Guidelines for Culturing the Japanese Medaka, *Oryzias latipes*



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# GUIDELINES FOR CULTURING THE JAPANESE MEDAKA, ORYZIAS LATIPES

BY

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## FOREWORD

This manual is a description of culture techniques for the Japanese medaka that have been developed over the last five years at the Environmental Research Laboratory-Duluth. These methods are presented as a culture system that works, not as the only way to culture medaka. Many different culture methods exist, and it is up to the user to decide what modifications or combinations of methods are appropriate for a specific application. The report has been reviewed by the Environmental Research Laboratory-Duluth, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

This paper describes culture techniques for producing large numbers of all life stages of Japanese medaka, Oryzias latipes, for use in biological research. The biology of the medaka is described as it relates to culturing practices and the physical systems used to maintain a large culture. The physical systems include water delivery apparatus, tanks, incubation pans, lighting, spawning substrates and other useful tools. The biological section addresses water temperature, spawning ratios, embryo incubation, juvenile rearing, and larval and adult feeding.

ACKNOWLEDGEMENTS

Many people have been involved in the development of successful medaka cultures at ERL-Duluth. The authors would specifically like to thank Larry Herman, Rod Johnson, Joe Tietge, Nan Stokes, Duane Benoit, Gary Holcombe, Craig Wilson, Kathleen Jensen, Wes Smith, Jodi Collins, and Roger LePage.

Much of the credit for development of culture techniques for new species is due to the wealth of experience in fish culture resident in the staff at the Environmental Research Laboratory-Duluth. This collective knowledge has been invaluable.

## INTRODUCTION

The Japanese medaka, Oryzias latipes, has been used in various fields in biology, especially in developmental biology, genetics, and embryology (Yamamoto, 1975). Aquatic and environmental toxicologists have recently begun to explore use of the medaka as a sentinel or biomarker for environmental contamination (Grady, et al., 1991). At the U.S. EPA Environmental Research Laboratory in Duluth, Minnesota, the medaka is being evaluated in a multidisciplinary research program encompassing carcinogenicity testing, metabolism, neurotoxicity, reproductive toxicity, and comparative toxicity for both acute and chronic endpoints. The medaka is also being tested for future use in the development of EPA freshwater aquatic life criteria documents, and the detection of carcinogenic components in industrial effluents and contaminated sediments.

The culture techniques described here are based on years of experience in the large scale culture of fathead minnows at ERL-Duluth. The aquarium systems have been continually refined over 25 years by dozens of workers. The biological methods for rearing medaka have been developed over the last five years by numerous people. Some methods are employed because of their traditional success, and others because they are cheap, simple, or minimize labor. There is very little "hard data" on how the culture system works. It has grown along with the size of the medaka research effort, eventually being capable of supplying thousands of embryo, larval, or juvenile medaka each week for research purposes. Over

300 toxicology related experiments have been performed at ERL-Duluth using medaka from this culture system.

The medaka is an extremely adaptable organism, and will thrive under a wide variety of conditions. Indeed, this adaptability is a crucial quality that makes the medaka so useful as a research animal. Adult medaka measure from two to four cm long, are very hardy, and can be easily maintained at room temperature (Kirchen and West, 1976). This simplicity of care makes it possible to establish a small culture of fish in almost any laboratory. Since medaka breed readily in captivity, small cultures can provide enough embryos for some types of research. For large scale experiments requiring 1000 or more fish of all life stages, intensive culture techniques are necessary.

It is outside the scope of this paper to delineate specific ranges for all parameters involved in the successful culture of medaka. For basic information on culture systems, water quality parameters, and general fish culture guidelines, the reader is referred to works such as Aquarium Systems by Hawkins (1981), Aquaculture, by Bardach, et al. (1972), or Fish and Invertebrate Culture by Spotte (1970).

Japanese biologists have been using the medaka as an experimental animal for at least 70 years (Yamamoto, 1975). Yamamoto (1975) gives an exhaustive description of all aspects of medaka biology, and includes a large bibliography. Recent papers concerning the use of medaka in carcinogen assays include Ishikawa et al., 1984, Klaunig, et al., 1984, and Hyodo-Taguchi and Egami,

1985. A basic discussion of the care and development of the medaka can be found in Kirchen and West, 1976.

## CHAPTER 1

## PHYSICAL SYSTEMS

The physical systems are quite similar to those described for fathead minnows in Denny (1987). Figure 1 provides a generalized schematic of how the flow-through aquarium system works.

### 1.1 Tanks

The tanks are 57 liter (15 gallon) glass aquaria with standpipe drains adjusted to provide 20 cm of water depth. One hole (2.5 cm) is drilled in each tank bottom, a stopper with a hole is inserted, and a drain is fashioned through the hole in the plywood rack. This allows for approximately 40 liters of water per tank. The incoming water flows through the tanks and out the standpipe drain. The tanks, supported by racks of slotted angle iron and 1.9 cm (3/4 inch) plywood, are arranged in two tiers, in rows of 12 tanks. Two units are used for spawning, rearing, and holding research fish at ERL-D. This setup supports production of 3,000 - 4,000 embryos/week, along with rearing and holding space for thousands of fish of all life stages. Groups of future brood stock are held in four 285 l (75 gallon) flow-through tanks.

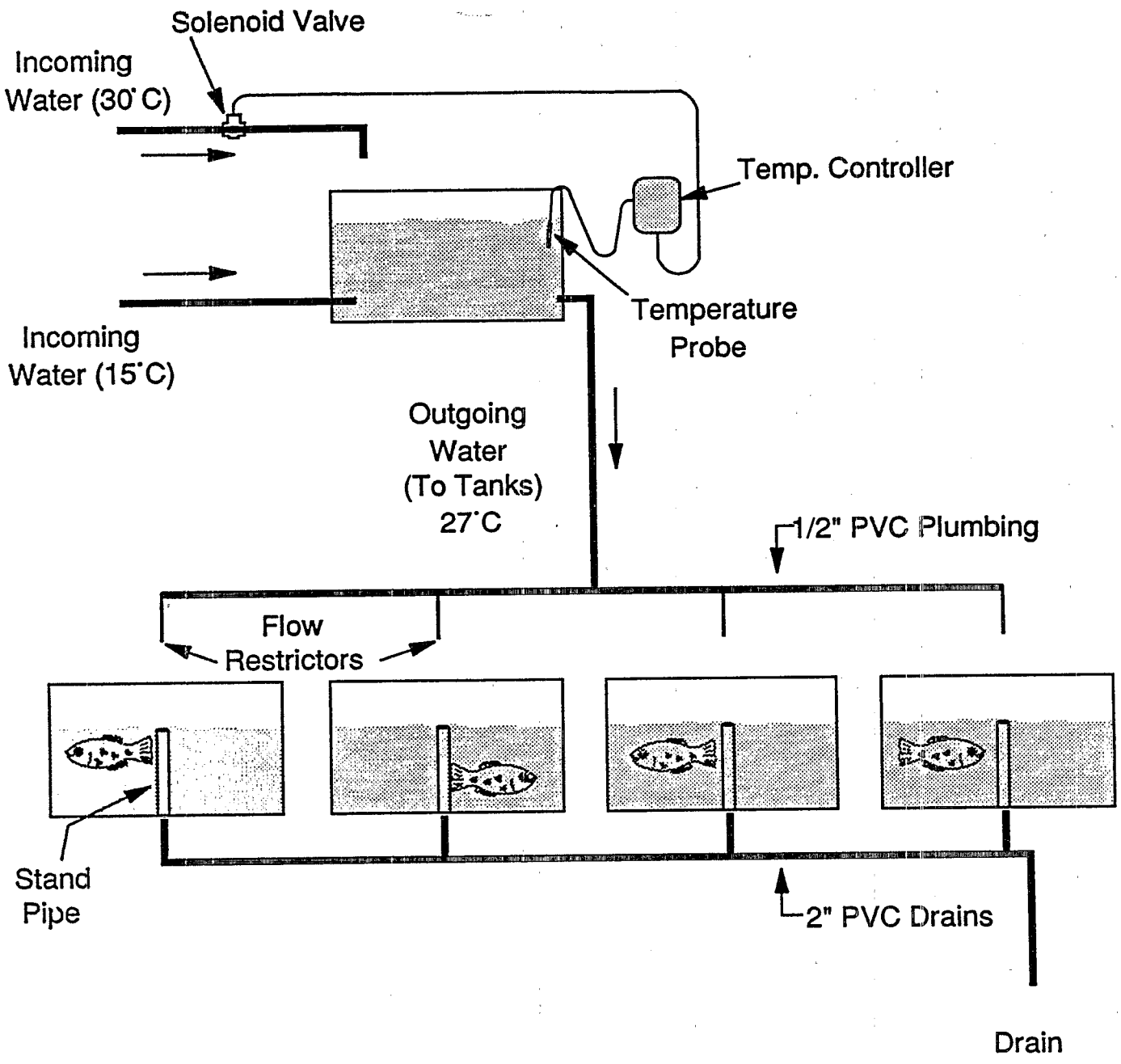


Figure 1. Temperature controlled, flow through culture apparatus.

## 1.2 Hatching Pans

Plastic dishpans, commonly available in department stores (e.g.: 53 cm x 40 cm x 12 cm deep) are used for embryo incubation. White pans provide the best background for viewing the newly hatched larvae. Pans are held in a temperature controlled water bath (27 °C), to decrease temperature shock due to fluctuation of room temperature. Incoming water (27 °C, 150 ml/min) flows in and through the hatching pan and out a screened drain. Temperature can also be maintained by using walk-in incubators, or individual aquarium heaters.

## 1.3 Water Supply

The two main criteria for water supplied to a culture facility are that it meets or approaches optimum conditions for the physiological needs of the fish, and that it be free of contaminants. It is generally desirable to use water from a spring, a well, or a controlled surface water with consistent water quality. Culture water should be similar to water used in testing. The source should be examined for contamination by pesticides, heavy metals, sulfides, disease vectors, or any other suspected contaminants. Filtration may be necessary if well water is used (Mount, 1971). Dechlorinated tap water from a municipal water supply should be used only as a last resort (Benoit, 1982). If the water is contaminated with fish pathogens, pass the water through an ultraviolet or similar sterilizer immediately before it enters the system (Allison and Hermanutz, 1977).

Water quality parameters such as hardness, alkalinity, and anions should fall within the following limits: hardness 40-300 mg/l (as CaCO3), and alkalinity slightly less than the hardness. The anions should be those found in a normal stream or lake. Avoid well or spring waters which have high levels of iron, silica, or sulfides not found in surface waters.

Research goals and design will dictate both the quality and quantity of water necessary to support medaka cultures. The ERL-D medaka culture system uses Lake Superior water with a pH range of 7.4-8.2, an alkalinity (as $CaCO_3$) of 42 mg/l and a total hardness of 45 mg/l. For a more detailed characterization of Lake Superior water see Biesinger and Christensen (1972).

## 1.4 ERL-D's Flow-Through System

The ERL-D water delivery system is a constant temperature, flow-through system (see Fig. 1, Sec 1.1). It is gravity fed, with custom welded stainless steel mixing boxes (46 cm x 28 cm x 40 cm deep) positioned on a shelf approximately 1 m overhead. Lake Superior water warmed to 15°C flows through a toilet tank valve to maintain water level in the headbox. Water heated to 30°C flows through a solenoid valve positioned over the headbox. To achieve the desired constant temperature, a temperature probe suspended in the headbox feeds into a solid-state temperature controller (Syrett and Dawson, 1975). Headbox temperature must be maintained at 28 - 29°C to provide 26 - 27°C in the tanks, to counteract heat loss to room air. This basic water delivery system can supply most

experimental water temperatures, depending only on the temperature of the water supply to the float and solenoid valves.

Agitators or airstones must be used in the headbox to assure complete mixing and to prevent supersaturation of gases caused by heating water. Water flows from the headbox through 1.27 cm (1/2 inch), threaded (not glued) polyvinyl chloride (PVC) pipe. This type of pipe (schedule 80), is available commercially. The outlet is located on the side of the headbox, about 2 - 3 cm from the bottom, to prevent sediment from clogging the outflow. Detailed instructions for the design for the mixing boxes, electronic relays, etc., are contained in Syrett and Dawson, 1972, 1975, and McCormick and Syrett, 1970.

Water flows into the tanks through a 1/2 inch PVC pipe manifold. At one or two points on the manifold an open ended pipe is extended upward above the water level in the headbox to eliminate possible air blockage. Above each tank is placed a tee with a 1/2 inch to 3/8 inch reducer attached. Into the reducer a 3 ml disposable syringe barrel is glued using silicone glue. Hypodermic needles or capillary tubing of different gauges can be attached to the syringe to provide different flow rates into the tanks. ERL-Duluth's 40 liter tanks are supplied with 200 ml of fresh water per minute, providing 7-8 turnovers each 24 hours. Grady, et al. (1991), reported success using 100 ml/min flows with 75 liter tanks. Again, space and other physical constraints may modify the flow rate or nature of the aquarium system.

Medaka can be maintained in static or recirculating systems, but flow-through systems allow higher stocking densities and feeding rates, while problems associated with disease organisms and waste products are reduced.

Recirculating systems often consist of aquaria at table-top level for the fish, and an aquarium on the floor that acts as a trickling filter for the drain water from the fish tanks. This filter can be made of any nontoxic, high surface area material such as crushed coral, pea gravel, or tower packing. Water can be pumped from a sump up to a headbox above the fish tanks, from whence it flows back into the tanks. Nitrifying bacteria in these filters convert ammonia to nitrate. Ammonia must be monitored closely in static or recirculating systems. The chronic effects threshold for fathead minnows exposed to ammonia, based on histological damage, is estimated to be .15 mg/l (Thurston, et al. 1986). For a discussion of filtration for recirculating systems, see Hawkins (1981).

## 1.5 Photoperiod

The relationship between photoperiod and reproductive activity in the medaka remains unclear. Papers addressing the issue (Yoshioka, 1963, Awaji and Hanyu, 1989) report conflicting results depending on geographical strain or maturational stage. We have found that a 16 light/8 dark regime stimulates reproduction, while a 8L/16D regime halts reproduction. All medaka in the ERL-D culture unit are maintained under the 16L/8D regime. Four foot

fluorescent tubes that simulate sunlight (e.g.: Duro-test Vita-lite, Sylvania Gro-lux or Gro-and-sho, or GE Chroma 50) are used. Light intensities at the water surface average 400 - 500 lux. This stimulates the growth of periphyton which the fish are observed to actively graze upon. If a strictly defined diet is critical to research goals, it is necessary to keep the tanks scrupulously clean to deny grazing opportunity. Medaka will also spawn under cool white lighting and shorter light periods.

## 1.6 Construction materials

Culture water should not contact brass, copper, lead, galvanized metal, or natural rubber. Items made of neoprene rubber or other materials listed above should not be used unless it has been shown that their use will not adversely affect either survival or growth of embryos and larvae of the test species (ASTM, 1990). Glass, teflon, stainless steel and PVC are acceptable materials.

## 1.7 Temperature control

Temperature control is accomplished with paired controllers, connected to temperature probes and solenoid valves as described in Section 1.4. A timer set to operate from 5 a.m. to 9 p.m. keeps the temperature at 28°C during the daylight period. During the dark period a temperature of 22°C is maintained. This fluctuating temperature regime helps to stimulate reproduction (Kirchen and West, 1976). Fail-safe devices such as temperature recorders,

shut-off solenoids, etc. are in order if mechanical malfunction can result in rapid change in water temperature.

## 1.8 Aeration

Mechanical aeration provided by air-lines in individual tanks has been avoided in the ERL-D culture system because female medaka will deposit embryos on the air-line rather than on the embryo collection substrate. In addition, vigorous aeration can damage newly hatched larvae. Also, aeration is usually not as necessary in high turnover, flow-through situations; however, the traditional aeration system consisting of an air pump, air-line, and air-stone is suitable for static systems. Embryos can be collected from the air-lines if necessary, and aeration can be reduced for young larvae.

## 1.9 Spawning Substrates

Spawning substrates essentially take the place of the roots of aquatic plants, which are the preferred natural sites for egg deposition for the medaka (Yamamoto, 1975).

Some criteria necessary for a useful spawning substrate are: They must be 1) acceptable to fish,

2) easy to handle (cleaning, storage, etc.)

3) non-toxic

4) inexpensive, readily available

The ERL-D system uses cylindrical foam aquarium filter cartridges for spawning substrates (see Fig. 2). These

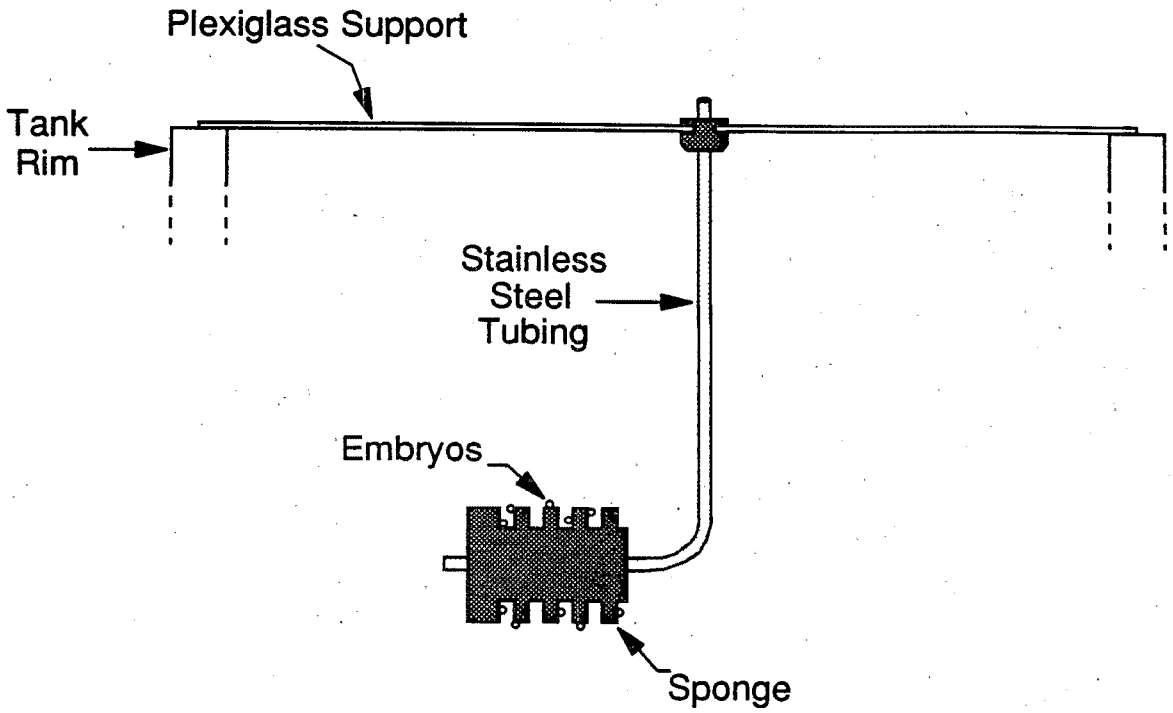


Figure 2 Spawning substrate, suspended in tank.

cylindrical sponges which are approximately 100 mm long and 50 mm in diameter, are available from Carolina Biological Supply. Though not similar to any natural substrate, the fish acclimate to these sponges, and will readily brush eggs off onto them. This method of obtaining embryos was modified from that used at The Gulf Coast Research Laboratory in Ocean Springs, Mississippi (William Walker, personal communication).

The sponges are suspended below the water surface in each spawning tank by means of an elbow constructed of 9mm diameter stainless steel tubing. The tubing is held in place by pressing it into a slot in a plexiglass support that lies across the top of the tank. Many other types of substrates and hanging methods are possible as long as they fit the above mentioned criteria. The substrates are moved directly from the spawning tanks to the hatching pans. This process avoids handling embryos, reducing labor and damage to the embryos.

## 1.10 Disturbance

Fish should be shielded from continual or drastic disturbance. Avoid construction noises, continual human presence, and extraneous lights that might alter the photoperiod. This is particularly true of spawning adults; excessive disturbance or activity can result in reduced embryo deposition. On the other hand, fish in strict isolation become hypersensitive to people and may take time to acclimate to increased levels of human activity.

## 1.11 Tank Cleaning

The four inch wide razor scrapers used to remove wallpaper are an excellent tank cleaning tool, and are available in hardware stores. After scraping the tanks, the debris should be siphoned away to remove potential disease substrate and to improve observation. If there is a tendency to vacuum up fish during the siphoning stage, siphon the residue into a screened pan that allows overflow, yet catches any errant fish.

Many culturists allow light growths of algae, rotifers, etc., to remain as a dietary supplement for the fish. This may not be acceptable for some specific research needs: e.g. maintenance of a strictly defined diet. Excessive growths of blue-green algae or fungus must be removed under any regime. An appropriate cleaning schedule can be determined depending on water source and feeding regime.

# CHAPTER 2

# BIOLOGICAL SYSTEMS

Four life stages are used for testing at ERL-Duluth - embryos, larvae, juveniles, and adults. The culture system is designed to produce large numbers (1,000+) of each life stage, depending on testing demands. Research projects that require only one life stage could be supplied by smaller and less labor intensive culture systems. For continuous, full life cycle culture, plans must be made to set aside a randomly selected group of fish from each generation to use for future breeding stock. Selection for

specific brood stock characteristics can also be employed, depending on intended research use.

## 2.1 Brood Stock

Medaka cultures were initiated at ERL-Duluth in April, 1986, using two breeding sets (4 males/6 females per set), from Carolina Biological Supply. They were derived from a stock of 2000 cultivated orange strain adults obtained by Carolina Biological Supply in 1970 from a wholesaler in Tokyo, Japan (R. V. Kirchen 1990, pers. comm.). Four more breeding sets were added to the ERL-Duluth gene pool in Sept, 1986. These original 60 fish have been used to produce a standing pool of 48 breeding sets. Current production consists of 3,000 - 4,000 embryos per week. Individual lots of larvae, juveniles, and adults (1,000 or more), are provided to researchers on request, or set aside for future brood stock.

Yamamoto (1975) describes 13 distinct genetic strains of the medaka: brown, blue, orange-red, variegated orange-red, white, variegated white, gray, pale, cream, milky, albino, ankylosed, and lordotic. He states, "The origin of orange-red and other color varieties of cultivated stocks is wrapped in a shroud of mist. The orange-red fishes have been painted by Ukiyoe artists of the Yedo era, so the race must have arisen by mutation from the wild type more than a few hundred years ago. The orange-red stock and a few other varieties have since been kept by goldfish breeders. These "himedaka" are available at goldfish shops and night stalls in any large city in Japan." (Yamamoto, 1975). This stable strain history

and easy availability may explain why the orange-red or himedaka is the strain most often used in research.

Inbred strains of medaka have been established through sibling matings, and some have been maintained in the laboratory for over 30 generations (Hyodo-Taguchi and Egami, 1985). The authors reported that during the inbreeding, reproductive potential was reduced or the mortality of the fish was high in many pedigrees. Two of the orange-red and three of the brown (wild) strains were successfully inbred by full sister-brother matings for 22 successive generations from 1974 to 1979.

The ability to maintain viable inbred strains in the laboratory may allow development of specific strains of medaka for specific toxicological research issues, much like inbred strains of mice (Shimkin, 1974). Development of an F1 hybrid strain may be a logical next step in the development of a medaka model. Montesano et al. (1986) states: "Although we know that the choice of strain may be important in a particular bioassay, we have in general insufficient knowledge to permit reliable prediction in advance of which strain to prefer. Indeed, there is not even general agreement as to whether inbred, F1 hybrid or outbred animals are usually to be preferred." Given that medaka genetics have been well studied (Yamamoto, 1975), it seems that the medaka model holds promise for the development of strains paralleling those of the mouse, rat, or Syrian hamster models.

## 2.2 Selecting spawning fish

Spawning sets of 4 males and 6 females each (Kirchen and West, 1976) are selected randomly at ERL-Duluth from a population of approximately 1,500 adult fish maintained in four 300 liter (75 gallon) aquaria. These breeding sets are established when the fish are 3 to 4 months old, and are replaced periodically as egg production drops. Commonly, the age range of the oldest set of spawning fish is 14-15 months. The ERL-D culture system has two banks of spawning tanks, with twelve tanks each. While one bank is being replaced, the other bank continues to supply embryos. One bank is replaced every 4 - 6 months.

When spawners are being selected, a random sample of 50-75 mature fish is placed into a one gallon aquarium. A contrasting background (i.e. black paper or wrinkled aluminum foil) is placed behind the tank. Side lighting allows for the easiest viewing of the translucent fins, which are used to differentiate the sexes. After waiting a few minutes for the fish to settle down, a small net is used to select and separate males and females.

Sexually mature medaka exhibit dimorphism which is most readily apparent in the dorsal and anal fins. Males have a small notch in the posterior margin of the dorsal fin and have a sail-like, transparent anal fin which is pointed at the distal posterior edge. Females have no notch in the dorsal fin and have a more compact anal fin which is rounded on the margins. Gravid females are easily spotted after spawning by the clutch of eggs attached to the oviduct pore.

When females are sexually mature there is an enlargement or swelling of the abdominal region caused by the presence of unfertilized eggs and mature reproductive tissues. Males retain their original fusiform shape throughout their life cycle. In healthy fish, dimorphism is usually discernable at approximately 2 months of age. Oka (1931), and Egami (1954) discuss sexual dimorphism of the medaka in detail.

## 2.3 Spawning

Because medaka are polygamous, Kirchen and West (1976) suggest a breeding ratio of three females to two males. Original breeding sets consisting of six females/four males were maintained in individual 40 liter tanks. This low stocking density works well, allowing collection of 3 - 4 thousand embryos on demand from 36 breeding sets. Low density decreases the likelihood of disease, minimizes problems with ammonia and other waste build-up, and allows for easier inspection, counting, etc. of the breeding stock. Medaka can be spawned at much higher densities, however, and recent experiments have shown that increasing the density from 10 to 20 or 40 breeding adults per tank results in increased embryo output (See Table 1).

Table 1. Mean number of embryos collected using one, two, or four breeding sets per tank. Numbers represent embryos voluntarily deposited on spawning sponges, not total reproductive output.

| # OF SPAWNING SETS | MEAN # OF EMBRYOS | STD. ERR. OF MEAN |
|---|---|---|
| 1 (control) | 87.4 | 5.2 |
| 2 | 192.7* | 12.6 |
| 4 | 365.0* | 16.9 |

* significant difference from control, p=0.05, TOXSTAT (program), ANOVA, Tukey's method of multiple comparisons.

Grady, et al. (1991), report breeding success with densities of 8 - 15 adult medaka per gallon of water. Stocking density and maintenance of water quality at higher densities are system dependent. Each culturist must experiment and work within the limitations of the available system.

Female medaka lay their eggs immediately after ovulation, which takes place almost invariably in the early morning. The entire courtship and fertilization process takes less than 60 seconds (Robinson and Rugh, 1943).

Yamamoto (1975) describes egg deposition as follows: "After the ova are expelled *en masse* at the time of mating, a cluster of eggs remains attached to the belly of the female for some hours, suspended from the oviduct pore by fine threads attached to the chorion. Finally, the egg mass is detached by the action of the female in swimming and contacting the roots of water plants, or if there is no vegetation by contacting the bottom of the container." In the ERL-D laboratory cultures, the spawning sponges described in

a previous section act as surrogates for deposition sites on aquatic plants.

## 2.4 Embryo Collection and Incubation

Embryos are collected from spawning adults once a week. More frequent collections are possible, and are undertaken for research projects with special demands. Usually the spawning substrates are placed in the tanks on Thursday mornings and allowed to remain for 24-28 hours. On Friday afternoon, the sponges are removed and the number of embryos deposited per sponge is recorded by tank number. A tack can be used to mark a spot on the cylindrical sponge substrate: counting from the tack, the sponge can be rotated 360 degrees, counting or estimating the number of embryos. Inviable embryos are removed with tweezers. At this stage, embryos can be provided for research projects designed to begin with embryos. Embryos can be segregated by developmental stage at this point if required (Kirchen and West, 1976).

Embryos are usually incubated as a group to provide lots of larvae of known age for initiating exposures (e.g.: 800+ for an early life stage test). Embryos are incubated on the substrates, in flow-through hatching pans, as described in section 1.2. At 27°C to 28°C hatching of the embryos will begin in about 7 days. By day 10, 95% of the embryos will have hatched. This hatching period can be modified by sorting embryos by developmental stage, by increasing incubation temperature to 28°C, or by physical manipulation of the embryos (Benoit et al., 1991). The least labor

intensive method is to simply allow the eggs to hatch undisturbed during the 7-10 day period and to pick off fungused embryos as they become visible. This is the method employed in the ERL-D culture system. On day 10, sufficient numbers will have hatched, so that larvae can be provided for testing, designated as <72 hours post hatch. One of the best tools for handling larvae is a large bore 50 ml volumetric pipette. Larvae to be counted can be pipetted from the hatching pan into a beaker in small lots of 10-15.

At times, female medaka will carry embryos for 1-2 days rather than deposit them, especially if a suitable substrate is not available. When a substrate is made available, the fish will then deposit these older embryos. Most lots of embryos are made up of 24 and 48 hour old embryos. Even if new substrates are provided daily, some fish will retain their embryos. If embryos of exact known age are required, observation and stripping of individual females may be in order.

Stripping embryos is not difficult, and is a standard technique in many Japanese laboratories (Tadakoro, pers. comm., 1989). Individual females carrying embryos are netted from the tank and placed on a wet towel. The embryos can be quickly brushed or picked off with a tweezer, taking care to point the sharp tips away from the abdomen (if the fish flips, the sharp tips could injure the abdomen). The female can be returned to the tank by lifting the towel and gently flopping the fish into the net. With practice, and especially with two people, this can be a quick, efficient operation, which does not damage the fish. Often,

females stripped in this manner will spawn again the next day. Stripped embryos can be segregated by developmental stage under a dissecting microscope (Benoit, et al., 1991).

## 2.5 Juvenile Rearing

Larvae not required for testing at this stage are stocked at a density of 200 larvae per 40 liter tank and reared to 30 days of age. Higher stocking densities can be used, but decreased survival and increased variability in size of juveniles can result from overstocking. These juveniles can satisfy testing protocols requiring the juvenile life stage. Larvae are stocked in this manner weekly, to provide a steady supply of juvenile fish for testing. Random lots of juveniles not used in testing are stocked at a density of 400-600 per 75 gallon tank and grown to maturity (3 - 4 months) for use as future breeding stock.

## 2.6 Feeding

Medaka have been maintained on a variety of foods: e.g.; Tubifex and Tetra-min (Awaji and Hanyu, 1989), Tubifex, Daphnia, and dried fish food (Robinson and Rugh, 1943), commercial tropical fish food, mosquito larvae, Euchytrea, and Artemia (Kirchen and West, 1976). In the ERL-Duluth culture system, newly hatched Artemia nauplii are the preferred full life-cycle food, with the exception of the last few days before sacrifice for histological analysis. Dried food (Cordon flakes) is offered for one week at

the end of the grow out period. This clears the gut of Artemia which interferes with histological sectioning.

All life stages of medaka are fed live Artemia ad libitum, twice each week day, and once a day on weekends. Because the density of the Artemia slurry can be variable, it is important to carefully observe the amount fed. In general, the fish are fed the amount they can consume in 20 - 30 minutes. Close observation is critical for adjusting feeding rates. If food is rapidly exhausted, more is supplied. If uneaten food is observed on the bottom of the tank hours later, the ration is decreased at the next feeding. In flow-through systems with large tanks it is not necessary to rinse the brine shrimp. In static or low flow systems rinsing is desirable to avoid salt build-up.

Since larvae begin to feed at approximately 24-48 hours post-hatch, it is important that food be offered at this time. The gape size of larval medaka is approximately .32 - .36 mm, while the width of a newly hatched Artemia nauplius is about .20 - .24 mm. The length of a newly hatched Artemia nauplius is .44 - .64 mm (T. Roush, pers comm, 1989). Larval medaka therefore are able to ingest first instar Artemia nauplii, but only in one orientation. If Artemia are allowed to grow for too long post hatch (into second instar), they become too large for the medaka larvae to ingest. They also provide fewer nutrients to the fish, since they use up the nutrient rich yolk sac in respiration and growth. For these reasons, it is extremely important to harvest the Artemia and feed the fish immediately after the Artemia hatch (first instar) as the

fish are essentially getting their nutrients from the yolk sac of the young Artemia.

## 2.7 Nutritional Content of Artemia

Different geographical strains and brands of Artemia have widely varying nutrient and contaminant levels. It is critical that the strain being used be analyzed for contaminants as well as for proximate nutritional content. Parameters for the strain of Artemia currently in use at ERL-D (Biomarine, Hawthorne, CA) are given in the Appendix (Tables 2-6). The analyses were performed by Woodson-Tenent Labs in Memphis, TN.

Mouse and rat carcinogenicity assays utilize synthetic diets in which the levels of contaminants and nutrients are defined. The National Institute of Health recommends an open formula diet where contaminant levels and heat labile nutrients must meet acceptable levels (National Toxicology Program, 1984). The Artemia used at ERL-Duluth have been screened for a wide variety of chlorinated organic contaminants, and have been found to be below those levels recommended by the NTP (Table 2). Braun and Schoettger (1975) compared the levels of selected contaminants in a variety of fish foods. The levels of DDT, PCBs, HCB, dieldrin, and endrin were much lower in the Artemia nauplii than in the commercial dry fish diets.

The nutritional requirements of rats, mice, and other animals have been elucidated through removal and substitution experiments with various dietary components. Dietary requirements are known

for some freshwater fish species, particularly carp (Nose, 1979), catfish (Wilson et al., 1978) and rainbow trout (Walton, et al., 1984). Medaka, however, have not received the detailed attention required to define their nutritional requirements.

Tables 3 and 4 provide the proximate analysis and amino acid profile for the lot of Artemia currently being used at ERL-Duluth. In general, 30%-55% crude protein is required for growth in fish (Millikin, 1982; National Research Council, 1983). These Artemia contain about 61% protein (Table 3). On a dry weight basis, the sum of the amino acids equals about 59%, which approximates the protein content.

Table 3 shows that these Artemia contain 5.42% crude fiber. Leary and Lovell (1975) found that fish foods with more than 8% fiber depressed fish growth. In practical diets, more than 3-5% fiber will probably not benefit the fish, as it will only increase waste production (National Research Council, 1983).

Artemia also contain vitamin E, vitamin C, and carotenoids (Table 3). Vitamin E promotes growth and prevents muscular dystrophy. In addition, it prevents the formation of fatty livers. This is important in medaka research, as the liver is a major target organ for many carcinogens, and fatty livers can interfere with histological sectioning and pathology analysis.

Vitamin C is essential for collagen metabolism. Since fish generally can't synthesize vitamin C and it is water soluble, a constant supply is needed to prevent deficiency signs. Though the

minimum level required in the diet of the catfish is only 60 mg/kg, the Artemia diet far exceeds that with 3917 mg/kg.

The bright orange color of Artemia is imparted by the carotenoids B-carotene (Czeczuga, 1980) and canthaxanthin (Soejima et al., 1980). Poston et al. (1977) found that B-carotene has the potential for conversion to vitamin A in fish. High levels of canthaxanthin were found to hasten the onset of spawning and increase the fertilization rate in rainbow trout (Deufel, 1965). This suggests that the carotenoid content of Artemia could be beneficial to the medaka.

Table 4 provides the percentages of the various amino acids. When these values are compared to the recommended values for other fish species (Table 5), most essential amino acid levels are comparable, as they fall within the pooled ranges. The level of methionine (1.81%) in the Artemia was found to fall below the average of the pooled range (2.99%), but the dietary need for this amino acid can be compensated for by the high level of cystine (0.72%). This has been substantiated by studies that show that the presence of dietary cystine in channel catfish and rainbow trout decreases the amount of methionine needed for maximum growth (Harding et al., 1977; Kim et al., 1983). In addition, phenylalanine (1.99%) was found to be quite low, but tyrosine, a non-essential amino acid that can replace 50% of the phenylalanine requirement in channel catfish (Robinson et al., 1980), was found to be high (2.35%) (Table 4).

Table 6 lists the fatty acid composition of Biomarine Artemia. Fish are incapable of de-novo synthesis of linoleic, linolenic, eicosa-pentaenoic and docosahexaenoic fatty acids. Thus, these are most likely to be the fatty acids essential for fish growth and survival (Kanazawa, 1985). When the levels of these acids are compared to the recommended levels in carp food (Takeuchi and Watanabe, 1977) and rainbow trout food (Castell et al., 1972), linolenic acid in Artemia is higher and linoleic acid is lower.

The diet analysis indicates that no carbohydrates are present in the Biomarine brand Artemia. Although carnivorous fish have an obvious need for protein and fat in their diet, they have difficulty digesting carbohydrates (Cho and Kaushik, 1985). Digestible protein can, in turn, provide much of the energy yielding nutrients needed by the medaka.

Thiaminase, a thiamin destroying enzyme often found in raw fish, is not found in Artemia (Greig and Gnaedinger, 1971). In this respect, Artemia may be superior to commercial fish foods that contain unpasteurized fish products. A thiamin deficiency has been shown to produce dark coloration and mortality in catfish, as well as fin congestion, nervousness, and a fading of body color in carp (National Research Council, 1983).

## 2.8 Disease

Diseased fish, or fish that have been chemically treated for disease cannot be used in testing. The best way to deal with disease is to prevent it. This is best accomplished by using low

stocking densities, keeping tanks clean, and using a nutritionally adequate diet. Any lots of fish discovered to be diseased must be discarded and the tanks disinfected. Methods for detecting fish disease can be found in Warren (1981), and Post (1983).

A dilution of 3.8 ml/liter of household bleach is an effective, cheap disinfectant for tanks and tools. This provides a concentration of 200 ppm free chlorine. Contact time should be 1 hour. Disinfection should not be overused, in order to avoid the production of chlorinated organic residues. Rather, it should be used to prevent the spread of disease, or at major junctures in the culture scheme (e.g.: sterilizing tanks to set up new spawners). Chlorine can either be neutralized with sodium thiosulfate, or allowed to dissipate for 24 - 48 hours. Test kits are available to check for the presence of free chlorine. Any tanks or tools that have been chlorinated must be rinsed thoroughly with culture water before being used with fish.

Fungus is ubiquitous in the aquatic environment. Its presence should not be considered a valid reason to discard all associated embryos unless the labor involved in picking and sorting out the bad embryos is not worth the return at hatch.

## 2.9 Record Keeping

Records are kept on the numbers of embryos collected each week, so that depleted spawners can be identified and replaced. All mortalities in the culture unit should be recorded, and any fish showing evidence of tumors or other deformities should also be

recorded and preserved. Records must be maintained on the age of spawning fish, and the dates on which rearing tanks are stocked with larvae. Recording embryo deposition dates and larval hatch dates is critical to wise stock management. For example, the embryo collection date is recorded, and a date label is placed on the incubation pan. Ten days later is the larval hatch date. Thirty days post-hatch is the "juvenile" date. At 60 - 90 days post-hatch fish begin to reach sexual maturity. Recording and tracking the ages of all fish in culture will allow the most efficient use of different life stages for testing. Fish should be observed daily for abnormal appearance and behavior.

## 2.10 Summary

Though many aspects of medaka biology have received intensive scrutiny, the use of this species as a toxicological tool has opened numerous new areas for inquiry. Definition of nutritional requirements, as has been done for trout or catfish, will allow for development of refined, formulated diets. This will be critical in metabolism, comparative toxicology, and carcinogenicity testing. Application of genetic information to the development of specific strains for specific research applications is also fertile ground for new research. Development of disease free cultures, such as those for other fish species or mammalian research models is another issue that has not been addressed. The need for research in these areas will become more urgent as use of the medaka model becomes more widespread.

## APPENDIX

### Nutritional and contaminant tables for *Artemia*

Table 2. National Institute of Health's Limits for Contaminants in NIH-07 Diet for Mice, Compared to the Pesticide Screen of Biomarine *Artemia* Nauplii

| Pesticide | NIH-07 Diet for Mice (PPM) | *Artemia* Nauplii (PPM) |
|---|---|---|
| Hexachlorobenzene | 0.02 | <0.01 |
| BHC | 0.02 | <0.01 |
| Lindane | 0.02 | <0.01 |
| Heptachlor | 0.02 | <0.01 |
| Aldrin | - | 0.01 |
| Heptachlor Epoxide | 0.02 | <0.01 |
| DDE | 0.02 | <0.01 |
| Dieldrin | 0.02 | <0.01 |
| Endrin | 0.02 | <0.01 |
| DDD | 0.02 | <0.01 |
| DDT | 0.02 | <0.01 |
| Mirex | 0.02 | <0.01 |
| Methoxychlor | 0.05 | <0.01 |
| Chlordane | 0.05 | <0.01 |
| Toxaphene | 0.10 | <0.01 |
| PCB (total) | 0.20 | <0.01 |
| Diazinon | 0.20 | <0.01 |
| Methyl Parathion | 0.02 | <0.01 |
| Malathion | 0.50 | <0.01 |
| Ethyl Parathion | 0.02 | <0.01 |
| Ethion | 0.02 | <0.01 |
| Ronnel | 0.02 | <0.01 |

| Table 3. Nutritional Analysis of Biomarine brand Artemia Nauplii | |
|---|---|
| Test | Dry Basis |
| Moisture | 94. 46%* |
| Fat | 37.36% |
| Protein - Kjedahl | 61.19% |
| Fiber, crude | 5.42% |
| Ash | 5.23% |
| Calories (bomb calorimeter) | 578 cal/100g |
| Beta Carotene | 3.97 mg/kg |
| Vitamin C | 3917 mg/kg |
| Alpha Tocopherol (Vit E) | 954 IU/kg |
| Iodine - low levels | 3.61 mg/kg |

* wet weight basis

| Table 4. Amino Acid Profile of Biomarine Brand Artemia Nauplii | |
|---|---|
| Test | Percent (as % of total dry weight) |
| Tryptophan | 0.90 |
| Aspartic Acid | 5.78 |
| Threonine | 3.61 |
| Serine | 4.33 |
| Glutamic Acid | 9.21 |
| Proline | 3.97 |
| Glycine | 3.25 |
| Alanine | 2.53 |
| Cystine | 0.72 |
| Valine | 1.99 |
| Methionine | 1.81 |
| Isoleucine | 2.35 |
| Leucine | 4.15 |
| Tyrosine | 2.35 |
| Phenylalanine | 1.99 |
| Histidine | 1.44 |
| Lysine (total) | 3.97 |
| Arginine | 4.87 |

Table 5. Essential Amino Acids in *Artemia* Compared to Requirements for Other Fish Species

| Essential Amino Acid | Fish Species* | Range (%) | Mean (%) | % in *Artemia* |
|---|---|---|---|---|
| Arg | abcdefgh | 3.3-6.0 | 4.7 | 4.9 |
| His | abcde | 1.5-2.1 | 1.9 | 1.4 |
| Thr | a | 2.0-4.0 | 3.0 | 3.6 |
| Ile | acdej | 2.0-4.0 | 2.7 | 2.4 |
| Leu | acdej | 3.3-3.9 | 4.0 | 4.2 |
| Val | acdej | 2.6-4.0 | 3.4 | 2.0 |
| Lys | acdefgh | 3.7-6.1 | 4.9 | 4.0 |
| Met | acdefghi | 2.0-4.0 | 3.0 | 1.8 |
| Cys | acdefh | 0-1.0 | 0.3 | 0.7 |
| Phe | acde | 5.0-6.5 | 5.6 | 2.0 |
| Trp | de | 0.3-1.4 | 0.7 | 0.9 |

% = as percent of total protein in diet

* a-chinook salmon
b-coho salmon
c-common carp
d-Japanese eel
e-channel catfish
f-rainbow trout
g-gilthead bream
h-tilapia
i-sea bass
j-lake trout

| Table 6. Fatty Acid Composition of Biomarine Artemia Nauplii | | | |
|---|---|---|---|
| Fatty Acid | % Wet Weight | Fatty Acid | % Wet Weight |
| Caproic Acid (6:0) | ND | Stearic Acid (18:0) | 6.64 |
| Caprylic Acid (8:0) | ND | Oleic Acid (18:1) | 30.56 |
| Capric Acid (10:0) | ND | Linoleic Acid (18:2) | 6.38 |
| Undecanoic Acid (11:0) | ND | Linolenic Acid (18:3) | 24.47 |
| Lauric Acid (12:0) | ND | Octadecatetraenoic Acid (18:4) | 3.38 |
| Tridecanoic Acid (13:0) | ND | Nonadecanoic Acid (19:0) | ND |
| Myristic Acid (14:0) | 0.94 | Arachidic Acid (20:0) | ND |
| Myristoleic Acid (14:1) | 0.46 | Gadoleic Acid (20:1) | ND |
| Pentadecanoic Acid (15:0) | ND | Eicosadienoic Acid (20:2) | ND |
| Pentadecenoic Acid (15:1) | ND | Eicosatrienoic Acid (20:3) | ND |
| Palmitic Acid (16:0) | 15.26 | Arachidonic Acid (20:4) | ND |
| Palmitoleic Acid (16:1) | 5.02 | Eicosapentaenoic Acid (20:5) | 3.08 |
| Hexadecadienoic Acid (16:2) | 1.13 | Behenic Acid (22:0) | ND |
| Margaric Acid (17:0) | 0.86 | Erucic Acid (22:1) | ND |
| Margaroleic Acid (17:1) | ND | Docosadienoic Acid (22:2) | ND |

## References


Allison, D.T., and R.O. Hermanutz. 1977. Toxicity of diazinon to brook trout and fathead minnows. U.S. Environmental Protection Agency. EPA 600/3-77-060.

ASTM, 1990. Annual book of ASTM standards, Water and Environmental Technology: Pesticides, resource recovery, hazardous substances and oil spill response, waste disposal, biological effects. Volume II. 04 designation E-1241-88. American Society of Testing and Materials, Philadelphia, PA 19103-1187.

Awaji, M., and I. Hanyu. 1989. Temperature-photoperiod conditions necessary to begin the spawning season in wild type medaka. Nippon Suisan Gakkaishi (formerly Bull. Japan. Soc. Sci. Fish.) 55 (4), 747.

Bardach, J.E., et al. 1972. Aquaculture: the farming and husbandry of freshwater and marine organisms. Wiley-Interscience Press. 868 pp.

Benoit, D.A. 1982. User's guide for conducting life cycle chronic toxicity tests with fathead minnows, (Pimephales promelas). U.S. Environmental Protection Agency. EPA 600/8-81-011. 17 pp.

Benoit, D.A., et al. 1991. User's guide for conducting early life stage toxicity tests with Japanese medaka (Oryzias latipes) draft. US EPA Environmental Research Laboratory, Duluth, MN 55804.

Biesinger, K. E., and G. M. Christensen. 1972. Effects of various metals on survival, growth, reproduction, and metabolism of Daphnia magna. Jour. Fish. Res. Bd. Can. 29: 1691-1700.

Brauhn, J. L., and R. A. Schoettger. 1975. Acquisition and culture of research fish: rainbow trout, fathead minnows, channel catfish, and bluegills. US Env. Prot. Agcy. EPA-660/3-75-011. 45pp.

Castell, J. D. et al. 1972. Essential fatty acids in the diet of rainbow trout (Salmo gairdneri): Growth, feed conversion, and some gross deficiency symptoms. J. Nutr. 102: 77-86.

Cho, C. Y. and S.J. Kaushik. 1985. Effects of protein intake on metabolizable and net energy values in fish diets. 95-117. in: C. B. Cowey, A. M. Mackie, and J. G. Bell, eds. Nutrition and feeding in fish. Academic Press, Harcourt Brace Jovanovich. 489pp.

Czeczuga, B. 1980. Carotenoid content of Artemia eggs and vitality of young specimens of this crustacean. In: G. Persoone et al. eds. The brine shrimp Artemia. Vol. 2. Physiology, Biochemistry, and Molecular Biology. Universa Press, Wetteren, Belgium. 636 p.

Denny, J.S. 1987. Guidelines for the culture of fathead minnows, Pimephales promelas, for use in toxicity tests. U.S. Environmental Protection Agency. EPA 600/3-87/001. 42 pp.

Deufel, J. 1965. Pigmentierungsversuche mit canathanxin bei Regenbogenforellen. Arch. Fischerewiss. 16: 125-132.

Egami, N. 1954. Appearance of the male character in the regenerating and transplanted rays of the anal fin in females of the fish, Oryzias latipes, following treatment with methyldihydrotestosterone. J. Fac. Sci. Imp. Univ. Tokyo. 4. 271-280.

Grady, et al. 1991. Laboratory management and husbandry of the Japanese medaka. Lab Animal. 20(3). 22-28.

Greig, R. A. and R. H. Gnaedinger. 1971. Occurrence of thiaminase in some common aquatic animals of the U.S. and Canada. Spec. Sci. Rep. Fish., U.S. Dep. Commer. Natl. Mar. Fish. Serv. 631: 1-7.

Harding, D.E., O.W. Allen, and R.P. Wilson. 1977. Sulfur amino acid requirement of channel catfish: l-methionine and l-cysteine. J. Nutr. 107. 2031-2035.

Hawkins, A.D., ed. 1981. Aquarium Systems. Academic Press. 452 pp.

Hyodo-Taguchi, Y., and N. Egami. 1985. Establishment of inbred strains of the medaka, Oryzias latipes, and the usefulness of the strains for biomedical research. Zoo. Sci. 2(3). 305-316.

Ishikawa, T., P. Masahito, and S. Takayama. 1984. Usefulness of the medaka, Oryzias latipes, as a test animal: DNA repair processes in medaka exposed to carcinogens. 35-43. in: K.L. Hoover, ed. Use of small fish species in carcinogenicity testing. Nat'l. Inst. Hlth. Monograph #65.

Kanazawa, A. 1985. Essential fatty acid and lipid requirement of fish. 281-298. in: C.B Cowey, A.M. Mackie, and J.G. Bell, eds. Nutrition and feeding in fish. Academic press. 489pp.

Kim, K.I., T.B. Kayes, and C.H. Amundson. 1983. Protein and arginine requirements of rainbow trout. Fed. Proc. 43(3): 667.

Kirchen, R.V. 1990. Carolina Biological Supply, Burlington, NC. letter dated 1/11/90.

Kirchen, R.V., and W.R. West. 1976. The Japanese medaka, its care and development. Carolina Biological Supply Company, Burlington, NC. 36pp.

Klaunig. J.E., B.A. Barut, and P.J. Goldblatt. 1984. Preliminary studies on the usefulness of medaka, Oryzias latipes, embryos in

carcinogenicity testing. 155-173. in: K.L. Hoover, ed. Use of small fish species in carcinogenicity testing. Natl. Inst. Hlth. Monograph #65.

Leary, D. F., and R. T. Lovell. 1975. Value of fiber in production type diet for channel catfish. Trans. Am. Fish. Soc. 104: 328-332.

McCormick, J.H., and R.F.Syrett. 1970. A controlled temperature apparatus for fish egg incubation and fry rearing. manuscript. U.S. Environmental Protection Agency, Duluth, MN.

Millikin, M.R. 1982. Qualitative and quantitative nutrient requirements of fishes: a review. Fishery Bulletin. 80: 655-686.

Montesano, R., et al., eds. 1986. Long term and short term assays for carcinogens: a critical appraisal. IARC Sci. Pub. #83. Oxford Press, Oxford, UK.

Mount, D.I. 1971. Tentative plans for the design and operation of a fathead minnow stock culture unit. U.S. Environmental Protection Agency, Duluth, MN 55804. 7 pp.

National Research Council, 1983. Nutrient requirements of warmwater fishes and shellfishes. National Academy Press, Wash, DC. 102pp.

National Toxicology Program. 1984. General statement of work for the conduct of acute, fourteen day repeated dose, 90 day subchronic, and two year chronic studies in laboratory animals. National Toxicology Program, P.O. Box 12233, Research Triangle Park, NC 27709.

Nose, T. 1979. In: J.E. Halver and K. Tiews, eds. Finfish Nutrition and Fishfeed Technology". 145-156. Heenemann GmbH and Co., Berlin.

Oka, T. B. 1931. On the processes of the fin rays of the male of Oryzias latipes and other sex characters of this fish. J. Fac. Sci. Imp. Univ. Tokyo 4(2). 209-218.

Post, G.W. 1983. Textbook of fish health. TFH Publications. Neptune City, NJ. 288 pp.

Poston, H.A., et al. 1977. The effect of supplemental dietary amino acids, minerals, and vitamins on salmonids fed cataractogenic diets. Cornell. Vet. 67: 472-509.

Robinson, E.J., and R. Rugh. 1943. The reproductive processes of the fish, Oryzias latipes. Biol. Bull. 84. 115-125.

Robinson, E.H., R.P. Wilson, and W.E. Poe. 1980. Total aromatic amino acid requirement, phenylalanine requirement, and tyrosine replacement value for fingerling channel catfish. J. Nutr. 110. 1805-1812.

Roush, T. 1989. Personal communication. U.S. EPA Monticello Environmental Research Station, Monticello, MN 55362.

Shimkin, M.B. 1974. Species and strain selection. in: L. Goldberg, ed. Carcinogenesis testing of chemicals. CRC Press, Cleveland, OH.

Soejima, T., T. Katayama, and L. Simpson. 1980. International study on Artemia XII. The carotenoid composition of eight geographical strains of Artemia and the effect of diet on the carotenoid composition of Artemia. p. 613-622. In: The brine shrimp Artemia. Vol 2. Physiology, Biochemistry, Molecular Biology. Persoone, G., P. Sorgeloos, O. Roels, and E Jaspars, eds. Univera Press, Wetteren, Belgium. 636 p.

Spotte, S.H. 1970. Fish and Invertebrate Culture. Wiley-Interscience Press. 145 pp.

Syrett, R.F., and W.F. Dawson. 1972. An inexpensive electronic relay for precise water temperature control. Prog. Fish. Cult. 34(4). 241-242.

Syrett, R.F., and W.F. Dawson. 1975. An inexpensive solid state temperature controller. Prog. Fish. Cult. 37(3). 171-172.

Tadakoro, Hiroshi. 1989. personal communication. Chemical Inspection and Testing Institute. 19-14 Chuo-Machi, Kurume, Fukuoka, Japan 830.

Takeuchi, T., and T. Watanabe. 1977. Requirement of carp for essential fatty acids. Bull. Jpn. Soc. Fish. 43: 541-551.

Thurston, R. V., et al. 1986. Chronic toxicity of ammonia to fathead minnows. Trans. Am. Fish. Soc. 115. 196-207.

Walker, William. 1987. personal communication. Gulf Coast Research Laboratory, Ocean Springs, MS.

Walton, M.J., et al. 1984. The effects of dietary tryptophan levels on growth and metabolism of rainbow trout, Salmo gairdneri. Br. J. Nutr. 51. 279-287.

Warren, J.W. 1981. Diseases of hatchery fish. U.S. Fish and Wildlife Service, Region 3. Federal Building. Ft. Snelling. Twin Cities, MN. 91 pp.

Wilson, R.P. et al. 1978. Tryptophan and threonine requirement of fingerling channel catfish. J. Nutr. 108. 1595-1599.

Yamamoto, T. 1975. Series of stock culture in biological field. Medaka (Killifish) biology and strains. Keigaku Publishing Company, Tokyo, Japan. 365pp.

Yoshioka, H. 1963. On the effects of environmental factors upon the reproduction of fishes. Bull. Fac. Fish. Hokkaido Univ. 137-151.



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